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POTENTIAL ROLE OF MONOCYTE CHEMOATTRACTANT PROTEIN 1/JE IN MONOCYTE/MACROPHAGE-DEPENDENT IgA IMMUNE COMPLEX ALVEOLITIS IN THE RAT¹

MICHAEL L. JONES, MICHAEL S. MULLIGAN, CRAIG M. FLORY,
 PETER A. WARD, AND JEFFREY S. WARREN²

From the Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109-0602

We have examined the role of monocyte chemoattractant protein 1 (MCP 1) in the pathogenesis of monocyte/macrophage-dependent IgA immune complex alveolitis in the rat. Rat MCP 1 was cloned and expressed in order to facilitate analysis of its function in rat models of human disease. A cDNA library was constructed from rat pulmonary artery endothelial cells stimulated with TNF- α . The cDNA library was screened with synthetic oligonucleotide probes based on the recently published rat MCP 1 cDNA sequence. Among numerous MCP 1-positive clones, four full length (approximately 480 bp) cDNA were rescued, amplified by polymerase chain reaction, and ligated into a pJVT12 baculovirus transfer vector. *Spodoptera frugiperda* insect cells (Sf-21) infected with baculovirus recombinants (*Autographa californica* nuclear polyhedrosis virus) bearing properly oriented MCP 1 cDNA (AcMCP 1) directed the expression of unique peptides of 18, 21, and 23 kDa. Treatment of AcMCP 1-infected Sf-21 cells with tunicamycin resulted in reduced production of the 21- and 23-kDa proteins and an increase in 18- to 18-kDa products, the predicted size range of uncleaved and nonglycosylated rat MCP 1. Denatured and refolded 23-kDa and 21-kDa rat MCP 1 species exhibited dose-dependent monocyte-specific chemotactic activity at concentrations as low as 10^{-10} M whereas the 18-kDa species exhibited negligible activity. Antibodies that react with the 18-kDa, 21-kDa, and 23-kDa MCP 1 bands by Western immunoblot, block rat MCP 1-directed monocyte chemotaxis, and neutralize monocyte-specific chemotactic activity secreted by TNF-stimulated rat endothelial cells were raised in rabbits immunized with the 23-kDa MCP 1 species. Intravenous administration of anti-MCP 1 antibodies upon initiation of IgA immune complex lung injury resulted in a marked reduction in lung injury as measured by pulmonary vascular permeability, alveolar hemorrhage, and pulmonary monocyte/macrophage recruitment. These data suggest that MCP 1 may play an important role in the pathogenesis of monocyte/

macrophage-dependent IgA immune complex alveolitis in the rat.

Despite the putative importance of IgA in diseases such as IgA nephropathy, Henoch-Schönlein purpura, dermatitis herpetiformis, and some cases of SLE, little is known about the pathogenesis of IgA-triggered tissue injury. We previously described an IgA immune complex-mediated lung injury model in the rat (1, 2). In contrast to various models of IgG immune complex-mediated tissue injury, IgA-induced injury develops fully in neutrophil-depleted rats (2). IgA immune complex lung injury requires an intact C system and is oxygen radical mediated (1, 2). Ultrastructural cytochemical analysis suggests that local production of H_2O_2 by mononuclear phagocytes is an important effector mechanism in IgA lung injury (3). In IgA lung injury monocytes and macrophages are recruited into the parenchyma and can be retrieved in BAL³ fluid during the development of injury. In contrast to IgG immune complex lung injury, in which locally produced TNF mediates neutrophil recruitment, negligible TNF activity can be detected in the BAL fluid of rats with IgA lung injury (4). The mechanisms through which monocytes and macrophages are recruited into the lungs of rats with evolving IgA immune complex-triggered alveolitis are unknown. A potential mediator of monocyte and macrophage recruitment is the monocyte chemoattractant, MCP 1.

MCP 1, known also as monocyte chemotactic and activating factor, is now known to be identical to the murine JE gene product (5). JE was described in 1983 as a gene that is expressed in mouse 3T3 fibroblasts after stimulation with platelet-derived growth factor (6). Although the JE gene product was not identified until later, its cellular sources and the stimuli promoting its expression were studied extensively (reviewed in Reference 7). MCP 1 is secreted either constitutively, or after induction with mitogens, cytokines, or growth factors, in a variety of cell types including lymphocytes, fibroblasts, endothelium, smooth muscle, and several tumor cell lines (reviewed in Reference 7). In vitro studies indicate that MCP 1 can enhance the tumorigenic activity of monocytes against several lines of tumor cells and that it is a potent chemotactic factor for monocytes (7-10).

Although in vitro studies have provided invaluable in-

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² Address correspondence and reprint requests to Jeffrey S. Warren, M.D., Department of Pathology, The University of Michigan Medical School, 1301 Catherine Street, Box 0602, Ann Arbor, MI 48109-0602.

³ Abbreviations used in this paper: BAL, bronchoalveolar lavage; MCP 1, monocyte chemoattractant protein 1; PCR, polymerase chain reaction; Sf-21, *Spodoptera frugiperda*-21; AcNPV, *Autographa californica* nuclear polyhedrosis virus; AcMCP 1, *Autographa californica* MCP 1; T-TBS, Tween-Tris-buffered saline; mol, multiplicity of infection; DNP-BSA, dinitrophenol-conjugated BSA.

sights into the potential functions of MCP 1, little is known about its role in physiologic or pathologic processes. The *in vitro* activities ascribed to MCP 1 suggest that it may be of fundamental importance as an inducer of the monocyte/macrophage-rich lesions that are characteristic of such pathologic processes as atherosclerosis, chronic inflammation, infiltration of tumors by monocytes, and granulomatous inflammation. Indeed, recent immunohistochemical and *in situ* hybridization analyses have revealed MCP 1 expression within macrophage-rich foci of atherosclerotic lesions in human and rabbit arteries (11, 12). Northern hybridization analyses of whole rat lungs containing glucan-induced granulomas indicate that MCP 1 gene expression is up-regulated during granuloma formation (13). Analyses of the biologic function of MCP 1 in such processes have not yet been carried out. In order to study the functional role of this mediator in rat models of human disease we cloned and expressed rat MCP 1.

In this study, we addressed the pathophysiologic role of MCP 1 in IgA immune complex-induced lung injury in the rat. This is an ideal model because lung injury is mediated by monocytes and macrophages and can be quantitated using several parameters. The data suggest that MCP 1 may play an important role in the pathogenesis of IgA immune complex alveolitis in the rat.

MATERIALS AND METHODS

Materials. *Spodoptera frugiperda* insect cells (Sf-21; B821-01), wild-type baculovirus stock (*Autographa californica* nuclear polyhedrosis virus; AcNPV), β -galactosidase recombinant stock (Ac-pJVTETZ), and AcNPV wild-type DNA were purchased from Invitrogen, San Diego, CA. The pJVTETZ transfer vector, (pBlueBac; a modification of pJVP10) (14), which contains the polyhedrin gene promoter, an *NheI* restriction site, an early transcriptase locus early promoter, and a β -galactosidase-coding region, was kindly provided by Dr. Chris Richardson (Biotechnical Research Institute, Montreal, Canada). Grace's insect cell culture medium was from GIBCO Laboratories, Grand Island, NY. FCS was from GIBCO. Streptomycin, penicillin, and fungizone were from Whittaker Bioproducts, Walkersville, MD. Sea plaque low melting agarose was from FMC Bioproducts, Rockland, ME. Restriction enzymes and other molecular biology materials were obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN; Bethesda Research Laboratories, Gaithersburg, MD; and New England Biolabs, Boston, MA. Synthetic oligodeoxynucleotides were prepared by the DNA Synthesis Facility (D. L. Oxender, Ph.D., Director), University of Michigan, Ann Arbor, MI.

Rat pulmonary artery endothelial cells. Rat pulmonary artery endothelial cells stimulated with human TNF- α were used for con-

Construction and screening of rat cDNA library. Total RNA was isolated by the guanidinium-isothiocyanate method (16) from rat pulmonary artery endothelial cells stimulated with human rTNF- α (200 U/ml) for 4 h. Poly (A) RNA was isolated by oligo(dT)-cellulose chromatography (16). cDNA synthesized by a modification of the Gubler and Hoffman method (17) was used to construct a library in pCDNA II (Invitrogen, San Diego, CA). pCDNA II (bearing an ampicillin-resistance gene) was electroporated into DH1aF⁺ *Escherichia coli* cells and the library amplified on LB + ampicillin (50 μ g/ml) plates. A [³²P]PCR-labeled oligodeoxynucleotide probe (5'-TAC AGC TTC TTT GGG ACA CCT GCT GCT GGT GAT-3'), complementary to positions 160-193 of the rat MCP 1 cDNA sequence provided by T. Yoshimura et al. (18), was used to screen the cDNA library by high density plaque hybridization (16). Hybridization to nitrocellulose filters was carried out at 37°C in 6X SSC, 5X Denhardt's solution, 0.05% sodium pyrophosphate, 1% SDS, 100 μ g/ml salmon sperm DNA, and 10⁶ dpm/ml probe. Filters were washed three times for 5 min with 6X SSC and 0.1% SDS at 37°C, three times for 30 min at 35°C, dried, and exposed to XAR-5 film (Kodak, Rochester, NY) at -70°C with intensifying screens. Appropriately sized MCP 1 cDNA were selected by successful PCR (Taq polymerase; Cetus Immune) amplification of cDNA using primer pairs that contained *NheI* restriction sites and bracketed the 5' and 3' termini of the rat MCP 1 cDNA sequence published by Yoshimura et al. (18). The primer sequences were: 5'-ATC^{NotI} AGC TAG CCT CCA CCA CTA TGC-3' and 3'-CTA AAC CTT ACA CTA^{NotI} CGA TCG GGT GG-5'.

Construction of the baculovirus transfer vector. The resulting MCP 1 cDNA PCR product of one of the selected full length rat MCP 1 clones (clone 2B) was cut with *NheI* according to manufacturer's instructions (New England Biolabs) and the DNA separated in 1% agarose (16). The resulting band was then cut out of the gel and purified using the following two-microfuge tube method. A hole was punched through the bottom of a 500- μ l tube containing angel hair and this tube (to which the DNA sample was added) was placed inside of a larger microfuge tube which was then centrifuged at 14,000 rpm for 5 min. The preparation was extracted in phenol/chloroform/indole acetic acid, the aqueous phase precipitated in cold ethanol, and the resulting DNA quantitated (16). The DNA pellet was then added to ligation mixes (with molar ratios of insert:vector of 0.5:1, 1:1, 2:1, and 4:1), which contained pJVTETZ that had been linearized with *NheI* and phosphatased (16). As a negative control pJVTETZ alone was subjected to the same set of conditions. The ligation products (and controls) were transfected into *E. coli* INV1aF⁺ (DH1 derivative) (Invitrogen) and amplified. MCP 1-positive colonies (see plaque hybridization protocol above) were picked, grown overnight in fungizone ampicillin growth medium, and subjected to DNA extraction (miniprep method) (16). Preparations containing appropriately sized MCP 1 cDNA inserts (\approx 480 bp) were then analyzed for proper insert orientation.

Proper orientation of the rat MCP 1 insert within the pJVTETZ transfer vector was assessed by using the oligonucleotide primer pairs that were complementary to a portion of the insert containing rat MCP 1 (near the 3'-end) and a portion of the transfer vector at a site adjacent to the 5' end of the MCP 1 insert. Using this method only properly oriented rat MCP 1 inserts would be expected to yield an appropriately sized (\approx 480 bp) PCR product. In addition, if insert orientation direction was random, approximately one-half of the insert-bearing vectors would be expected to yield a product. The primers were: "Vector" primer for pJVTETZ:

5'-GCC GGA TTA TTC ATA CCG TC-3' and
-34 -from *NheI* site - -15]

"Insert" primer for rat MCP 1: 3'-CTA AAC CTT ACA CTA CGA TCG GGT GG-5'.
[481 - 467]

struction of a cDNA library. The cells were extracted from rat pulmonary arteries by perfusion with microcarrier beads, characterized as endothelial, and maintained in culture as previously described by Ginsburg et al. (15). Cells (sixth passage) used for RNA extraction were grown to confluence in 150 cm² T-flasks (Corning Glass, Corning, NY). The day before RNA extraction the culture medium was removed, the monolayers washed twice with HBSS (GIBCO), and the medium replaced with RMP1 1640 (GIBCO) without serum, endothelial cell growth supplement, or heparin. Upon initiation of stimulation, endothelial monolayers were again washed and fresh medium without growth factors was added along with 200 U/ml rTNF- α (Cetus Immune, Emeryville, CA). After 4 h, culture medium was collected and RNA was prepared from the endothelium as described below. For molecular sizing of native rat MCP 1 serum-free conditioned medium was collected after 12 h.

Transfection of *S. frugiperda* cells and isolation of recombinant virus. The MCP 1-bearing transfer vector (AcMCP 1), amplified in *E. coli* INV1aF⁺, was mixed with wild-type AcNPV DNA (2 μ g/1 μ g) and co-precipitated with calcium phosphate (16). This mixture was used to transfect monolayers of Sf-21 cells maintained at a density of 2.0×10^6 cells/25-mm tissue culture dish. After 4 h the transfection mix was removed and the monolayers washed twice with Grace's medium. After 4 days, serial dilutions (10^{-1} to 10^{-6}) of culture supernatant were layered onto 75% confluent monolayers of Sf-21 cells, incubated for 1 h, washed, and overlaid with fresh Grace's medium containing 1% agarose (Sea plaque) and Blue-O-gal substrate (150 μ g/ml; GIBCO). The cultures were maintained for 4 days at 27°C by which time many cells contained occlusion bodies characteristic of the polyhedrin protein. Plaques devoid of occlusion bodies and positive for β -galactosidase expression (blue in the presence of Blue-O-

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gal) were purified and amplified by two successive rounds of plaque assays. *S. frugiperda* cells were grown and maintained at 27°C in Grace's insect medium (GIBCO) supplemented with 10% FCS, penicillin (50 U/ml), streptomycin (50 µg/ml), and fungizone (50 µg/ml) as described by Summers and Smith (19). For amplification of virus, wild-type baculovirus (AcNPV), or recombinant virus (AcMCP 1) was used to infect Sf-21 cells at a mol of 1.0 plaque-forming units/cell. Negative controls included wild-type virus (AcNPV), rMCP 1-negative virus (AcJVETL2), and noninfected Sf-21 cells.

Production of rat MCP 1 in Sf-21 cells. Recombinant virus stocks were grown from second passage virus in Sf-21 cells to obtain high titers ($>10^6$ plaque-forming units/ml). Pilot experiments were conducted to establish the optimal mol and the optimal time after infection to harvest the cells. For high level rat MCP 1 expression, suspension cultures of Sf-21 cells (10^6 cells/ml) were infected at mol = 1, and harvested 96 h after infection by centrifugation (700 × g). Pelleted cells were washed with Tris/Cl (50 mM; pH 7), 1 mM dithiothreitol and 250 mM sucrose, resuspended in 0.1 vol Tris/Cl (50 mM; pH 7), 1 mM dithiothreitol and 0.5 M NaCl, and then subjected to three freeze-thaw cycles in liquid nitrogen. Culture supernatants, cell pellet lysates (after DNA was sheared by three passes through a 26-gauge needle), and resuspended (0.1 vol; 50 mM Tris/Cl, pH 7, 1 mM dithiothreitol) cell pellets were used in subsequent analyses and preparations.

Characterization of AcMCP 1 expression products. Proteins expressed by AcMCP 1 and negative controls (uninfected Sf-21 cells, Sf-21 cells infected with wild-type baculovirus (AcNPV), and Sf-21 cells transfected with expression vector alone (AcJVETL2)) were characterized by SDS/PAGE under reducing conditions on 15.5% gels stained with Coomassie blue R or silver (20, 21). Analysis of MCP 1 glycosylation was carried out by SDS/PAGE using Sf-21 cells infected with AcMCP 1 and incubated in the presence or absence of tunicamycin (10 µg/ml). In vitro biologic activity (monocyte and neutrophil chemotaxis) was assessed as described below.

Chemotaxis assays. Monocyte and neutrophil chemotaxis assays were carried out in parallel in 48-well microchemotaxis chambers as described by Falk et al. (22). Cell suspensions (2.25×10^5 cells/well) were added to the top well of the chamber and permitted to migrate through 10 µm polycarbonate membranes (5 µm porosity for monocytes and 3 µm porosity for neutrophils) toward sample-bearing bottom chambers. After a 4-h incubation (37°C, 5% CO₂, humidified) membranes were removed and the nonmigrating cells wiped off. The membranes were then fixed for 10 min in absolute methanol, air dried, and stained for 30 min in 2% toluidine blue. The numbers of cells migrating through the membrane were counted in three random, 10-mm grids at 400 ×, with the mean ± SEM calculated for triplicate samples. Results are expressed as normalized values representing the percent of maximum FMLP (10^{-8} or 10^{-6} M; as indicated) positive control, minus negative controls (buffer alone). Peripheral blood human monocytes (80% to 85%) were isolated by centrifugation through Ficoll-Hypaque (Sigma, St. Louis, MO) and Sepacell-MN (Sepratech, Oklahoma City, OK) as described by Vissers et al. (23). Peripheral blood human neutrophils (91% to 96%) were isolated by centrifugation through Ficoll-hypaque as described by Boyum (24).

Protein solubilization and refolding. Freeze-thaw lysates from AcMCP 1-infected Sf-21 cells were subjected to preparative SDS-PAGE (12.5%). Unfixed, unstained 18-kDa, 21-kDa, and 29-kDa MCP 1 bands, and where indicated, a 44-kDa baculoviral protein band, were cut out and electroeluted into 8 M urea plus 10 mM 2-ME. This was dialyzed against 2000 volumes of (1 mM) 2-ME in HBSS (48 h, 4°C).

Rabbit polyclonal anti-MCP 1. Polyclonal rabbit anti-rat MCP 1 was raised against the 23-kDa MCP 1 in 3 kg New Zealand White rabbits (Charles River Laboratories, Wilmington, MA) immunized with MCP 1 (50 µg) emulsified in Hunter's TiterMax (CytRx, Norcross, GA) and boosted after 1 month with MCP 1 (25 µg). Where indicated, the resulting antiserum was affinity purified using a protein A Sepharose column (Sigma). Anti-MCP 1 serum was diluted 1/1 with PBS (100 mM phosphate, pH 8.0, and 150 mM NaCl) and applied slowly to the PBS-washed column. After extensive washing with PBS, the column was stripped with 100 mM sodium acetate buffer, pH 3.0. One milliliter fractions were collected in tubes containing 50 µl of 1 M Tris buffer, pH 8.0. The affinity-purified IgG fraction was then dialyzed against PBS.

Western immunoblot. Sf-21 cell pellet lysates were subjected to SDS/PAGE (12.5%) according to the method of Laemmli (20). The separated proteins were transblotted to nitrocellulose (0.45 µm; Bio-Rad, Richmond, CA) for 1 h at 12 V with a Gene-Screen apparatus (Ideal Scientific, Corvallis, OR). After transfer, the membrane was blocked with T-TBS (20 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.05% Tween 20) (v/v) containing 3% BSA, Cohn fraction V, (Sigma) for 2 h at room temperature. After removal of the blocking solution, the

blot was washed with T-TBS (5 min; three times). Primary antibody (affinity-purified rabbit anti-rat MCP 1; 1 mg/ml) was then added at final concentration of 50 µg/ml in T-TBS with 1% BSA (v/v) and incubated for 1 h. The primary antibody was decanted and the membrane washed as described above. After the final wash, secondary antibody (goat anti-rabbit IgG alkaline phosphatase-conjugated antibody (Bio-Rad) was added at a final dilution of 1/3000 in T-TBS with 1% BSA, and incubated for 1 h. The membrane was washed as above and the bands developed by addition of alkaline phosphatase substrate solution (5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium in 10 mM Tris; pH 9.5). Rainbow m.w. markers (Amersham, Arlington Heights, IL) were used to estimate m.w.

IgA antibodies and DNP/BSA Ag. Affinity-purified monoclonal IgA (MOPC 318) directed against DNP-BSA was purchased from Sigma. Dinitrophenol-conjugated BSA was prepared according to the method of Eisen (25). The resulting Ag preparation contained an average of 50 DNP groups/BSA molecule.

Rat IgA immune complex-induced alveolitis. Male Long-Evans pathogen-free rats (350 g; Charles River) were used for all studies. Intraperitoneal injections of ketamine (2.5 to 5.0 mg/100 g body weight) and sodium pentobarbital (5 mg/100 g body weight) were given for sedation and anesthesia. IgA immune complex lung injury was induced as previously described (1-4). Antibody solution (IgA anti-DNP/BSA; 400 µg) was instilled into the lungs through a tracheal cannula. In all cases, a final volume of 300 µl was instilled into the lungs. Ag (DNP-BSA; 3.3 mg) was injected i.v. Rats were killed at the indicated times, lung injury was quantitated, and BAL fluid was harvested. Anesthetized rats were exsanguinated through inferior vena caval section before removal of lungs, thus resulting in negligible contamination of lungs with blood. Pulmonary injury was quantitated by permeability, hemorrhage, and morphometric measurements. Permeability indices were calculated by comparing the leakage of ¹²⁵I-labeled bovine γ-globulin from the circulation into the lung to the ¹²⁵I-labeled colloid remaining in 1 ml of blood as previously described (1-4). Hemorrhage indices were calculated by comparing the leakage of ⁵¹Cr-labeled RBC from the circulation into the lung to the ⁵¹Cr-labeled RBC remaining in 1 ml of blood as previously described (1-4). Intravenous anti-MCP 1 or preimmune serum was infused at time zero.

Morphometric analysis of mononuclear phagocyte recruitment and alveolar hemorrhage. Lung samples (1 mm³) were excised from the peripheral aspect of whole lungs that had been fixed in 4% glutaraldehyde under constant pressure inflation (25 cm H₂O) (4). Samples were washed in 0.1 M cacodylate buffer (pH 7.3), embedded in 1 µm thick plastic sections, and stained with toluidine blue. Use of 1-mm³ samples from the peripheral aspects of inflated lung results in sections that contain no large bronchial structures. (Large bronchial structures are defined as muscular airways lined by respiratory epithelium). Plastic embedding (1 µm thick) allows very high morphologic resolution, thus allowing virtually all alveolar and alveolar septal cells to be easily identified. Morphometric analysis of mononuclear phagocyte recruitment and alveolar RBC (hemorrhage) was carried out by a pathologist (J. S. Warren) who was blinded to sample origin. For each condition, five samples were examined. In each sample, 45 to 60 randomly selected 40× microscopic fields (high power field) were analyzed.

Analysis of BAL fluid. Lung lavage contents for cell counts were collected using 5 ml of 37°C, serum-free RPMI 1640 (3, 4). At least 90% of the administered fluid was always recovered, centrifuged (400 × g; 7 min) to separate cells, and the cells were counted. There was no systematic difference in BAL fluid recovery between control and experimental groups of rats.

RESULTS

Rat MCP 1 cDNA cloning. Several studies have revealed that stimulation of human umbilical vein endothelial cells with TNF-α results in the expression of MCP 1 and the secretion of a monocyte-specific chemotactic protein (26-28). Accordingly, we constructed a cDNA library from rat pulmonary artery endothelial cells stimulated with human TNF-α and cloned a full length rat MCP 1 cDNA. The cDNA library, which contained greater than 1.2×10^6 ampicillin-resistant recombinants, was initially screened with an oligodeoxynucleotide probe that was complementary to positions 160-193 of the rat MCP cDNA sequence published by Yoshimura et al. (18). Among numerous MCP 1-positive colonies, four yielded products after PCR in which primers that bracketed the

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5' and 3' termini of the published rat MCP 1 cDNA open reading frame were employed (18). Plasmid DNA preparations prepared from these four clones each yielded fragments of approximately 500 to 700 bp after digestion with *Bam*HI and *Spe*I (polylinker sites in the pCDNA II cloning vector). One cDNA clone (clone 2B) was then amplified by PCR using primer pairs that contained *Nhe*I restriction sites and bracketed the 5' and 3' termini of the rat MCP 1 cDNA sequence.

Expression of rat MCP 1 in a baculovirus system. The baculovirus AcNPV is a helper-independent expression vector that has been used successfully to express several eukaryotic genes (reviewed in Reference 29). A rat MCP 1 expression vector was prepared by ligating the full length MCP 1 cDNA clone 2B (at varying insert:vector ratios) into pJVETLZ. Although successful ligations occurred at each insert:vector ratio employed (0.5:1, 1:1, 2:1, 4:1), a 1:1 ratio was used for this construction because it yielded the maximum number of products bearing single insert copies (data not shown). Proper orientation of the rat MCP 1 insert within the pJVETLZ transfer vector was assessed using the PCR with oligonucleotide primer pairs that were complementary to a portion of the insert containing rat MCP 1 (near the 3' end) and a portion of the transfer vector at a site adjacent to the 5' end of the MCP 1 insert. Of seven MCP 1-bearing transfer vectors, four yielded PCR products that indicated proper insert orientation (Fig. 1). Using this method only properly oriented MCP 1 inserts would be expected to yield an appropriately sized (481-bp) PCR product. In addition, if the insert orientation is random, approximately one-half of the insert-bearing vectors would be expected to yield a product.

Recombinant virus (AcMCP 1) was obtained by homologous *in vivo* recombination between wild-type virus (AcNPV) and the MCP 1-bearing transfer vector. Recombinant viruses were purified by two serial passages as described in *Methods*. Sf-21 cells were infected with rAcMCP 1 (third passage), recombinant virus lacking the MCP 1 insert (AcpJVETLZ), or wild-type virus (AcNPV). Uninfected Sf-21 cells were also included as an additional negative control. AcMCP 1 directed the expression of 18-kDa, 21-kDa, and 23-kDa bands that were not present in uninfected Sf-21 cells or in Sf-21 cells infected with either AcNPV or AcpJVETLZ (Fig. 2). The yield of rat

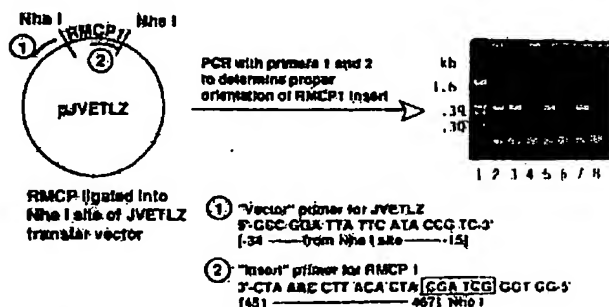


Figure 1. Proper orientation of rat MCP 1 (RMCP 1) in the pJVETLZ transfer vector. Lanes 2, 3, 5, and 7 contain rat MCP 1 insert ligated into pJVETLZ in the proper 5' → 3' orientation. The four properly oriented PCR products (lanes 2, 3, 5, and 7) migrate above the 0.39-kb size marker, at a location consistent with the expected 481-bp PCR product. PCR products were electrophoresed through 0.8% agarose and stained with ethidium bromide.

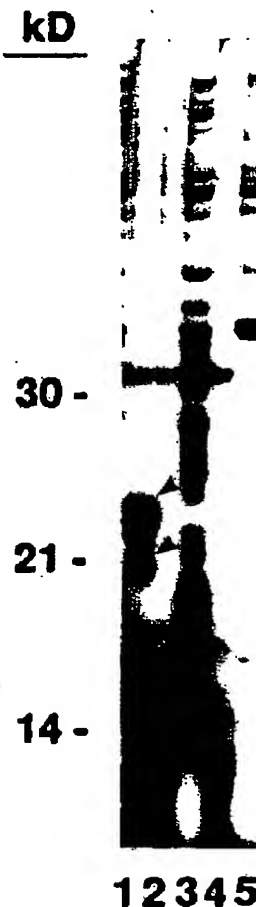


Figure 2. Expression of rat MCP 1 in Sf-21 cells. Sf-21 cells, grown at a density of 2.0×10^6 /25-mm culture dish, were infected with AcMCP 1 (lane 1), wild-type virus (AcNPV) (lanes 2 and 4), no virus (untreated Sf-21 cells) (lane 3), and recombinant virus lacking an MCP 1 insert (AcpJVETLZ) (lane 5). Proteins bands of 18 kDa, 21 kDa, and 23 kDa (arrows) are present only in Sf-21 cells infected with AcMCP 1. These cells were harvested 96 h after infection and lysed in 100 μ l 50-mM Tris/Cl, pH 7, and 1 mM dithiothreitol. After addition of SDS sample buffer, fractions were boiled for 5 min and 20 μ l of each applied to a 13.5% polyacrylamide gel (20). After electrophoresis, the gel was stained with silver according to the method of Wray et al. (21). Lane kd indicates m.w. markers in kilodaltons.

MCP 1 per 2×10^6 starting Sf-21 cells increased as a function of time over 96 h (data not shown). Over the same time period, Sf-21 cells infected with AcpJVETLZ expressed increasing concentrations of β -galactosidase, but no protein in the 18- to 23-kDa range. No new protein bands were observed in Sf-21 cells infected with AcNPV or in uninfected Sf-21 cells at any of the time points examined (data not shown). Essentially all of the stable rat MCP 1 (18 to 23 kDa protein) was recovered from solubilized Sf-21 cell lysates. The bulk of rat MCP 1 expressed by Sf-21 cells had a m.w. of 23 kDa. These data indicate that Sf-21 cells infected with AcMCP 1 direct the expression of proteins of 18 kDa, 21 kDa, and 23 kDa in a time-dependent manner over 96 h.

Characterization of rat rMCP 1. Based on an open reading frame of 444 bp (18), the predicted m.w. of unprocessed rat MCP 1 is 16.3 to 18.9 kDa. We employed tunicamycin to determine whether the 21-kDa and 23-

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kDa protein bands might represent glycosylated species of rat MCP 1. SDS/PAGE analysis of solubilized lysate pellets from Sf-21 cells incubated with tunicamycin revealed several major bands between 16 and 18 kDa and small residual 21-kDa and 23-kDa bands (Fig. 3). This observation suggests that Sf-21 cells infected with AcMCP 1 express variably glycosylated protein species.

Solubilized lysates from Sf-21 cells infected with AcMCP 1 exhibited negligible monocyte or neutrophil chemotactic activity in vitro (data not shown). Accordingly, we sought to solubilize and renature the protein of interest in the hope of producing biologically active rat MCP 1. Cell lysates from AcMCP 1-infected Sf-21 cells (96-h cultures) were subjected to SDS-PAGE. Individual bands (18-kDa, 21-kDa, 23 kDa, and irrelevant 44-kDa baculoviral protein) were cut out, electroeluted, and solubilized in 8 M urea plus 10 mM 2-ME followed by dialysis for 48 h against 2000 volumes of HBSS (4°C) containing 1 mM 2-ME. Renatured cell pellet lysates from AcMCP 1-

infected Sf-21 cells exhibited no neutrophil chemotactic activity (data not shown). The 23-kDa and 21-kDa rat MCP 1 species exhibited dose-dependent, monocyte-specific chemotactic activity, whereas the 18-kDa MCP 1 species exhibited little activity (Fig. 4). As shown in Figure 4, the irrelevant 44-kDa baculoviral protein exhibited no monocyte chemotactic activity.

Characterization of anti-rat MCP 1. Western immunoblot analysis of anti-MCP 1 revealed specific reactivity with the 18-kDa, 21-kDa, and 23-kDa rat MCP 1 species (Fig. 5). Anti-rat MCP 1 serum specifically blocked rat rMCP 1 (23 kDa)-induced monocyte chemotaxis in a dose-dependent manner with 95% blockade of MCP 1 (10^{-6} M) activity with undiluted anti-rat MCP 1 serum (Fig. 6). Equivalent concentrations of preimmune serum exhibited less than 10% blockade of MCP 1-mediated monocyte chemotaxis.

The capacity for anti-rat MCP 1 to neutralize native monocyte chemotactic activity was tested using TNF- α -

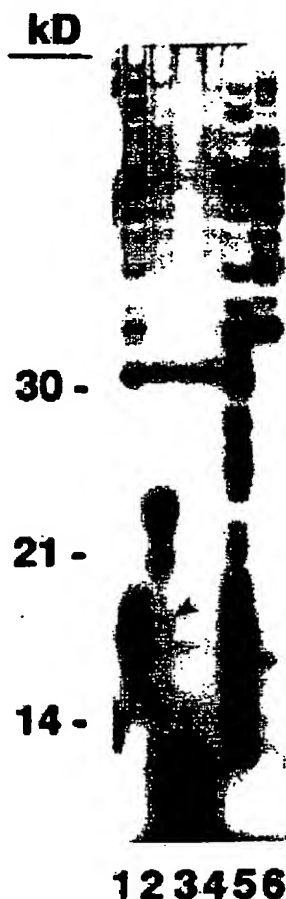


Figure 3. Effect of tunicamycin on rat MCP 1 expression. Addition of tunicamycin to Sf-21 cells infected with AcMCP 1 results in the production of a cluster of protein bands of approximately 16 to 18 kDa. Sf-21 cells, grown at a density of 2.0×10^6 /25-mm culture dish, were infected with AcMCP 1 in the presence of tunicamycin (10 μ g/ml) (lane 1), AcMCP 1 in the absence of tunicamycin (lane 2), wild-type virus (AcNPV) (lanes 3 and 4), no virus (uninfected Sf-21 cells) (lane 5), and recombinant virus lacking an MCP 1 insert (Ac Δ SVETL2) (lane 6). These cells were harvested at 96 h after infection and processed as described in the legend for Figure 2. As in Figure 2, there is a small amount of 18-kDa protein (arrow) produced by Sf-21 cells infected with AcMCP 1 (lane 2).

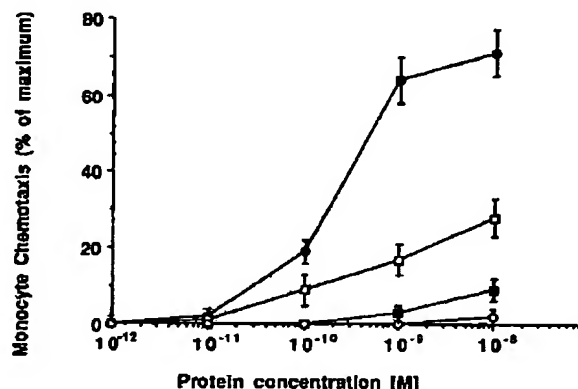


Figure 4. Renatured rat MCP 1 exhibits in vitro monocyte-specific chemotactic activity. The 23-kDa (—●—) and 21-kDa (—□—) MCP 1 species possess potent, dose-dependent monocyte chemotactic activity. The 18-kDa MCP 1 species (—■—) and an irrelevant 44-kDa viral protein (—○—) exhibit little and no monocyte chemotactic activity, respectively. The data (means \pm SEM) are expressed as percentage of maximum monocyte chemotactic response to FMLP (10^{-6} M). The average percentage of input monocytes that migrated in response to FMLP (10^{-6} M) was $27 \pm 4\%$. All samples were assayed in triplicate.

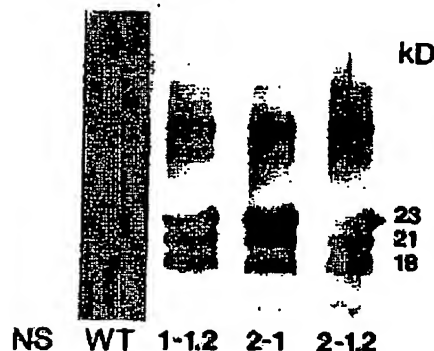


Figure 5. Rabbit polyclonal antibody raised against the 23-kDa rat MCP 1 reacts with the 18-kDa, 21-kDa, and 23-kDa rMCP 1 species. Western immunoblot was carried out as described in Materials and Methods. Preimmune serum from rabbit 2 (NS) did not react. Lane WT (wild type) represents reaction between anti-MCP 1 antiserum taken from rabbit 2 and reacted against cell pellet lysates from Sf-21 cells infected with AcNPV. Lanes 1-1.2, 2-1, and 2-1.2 represent anti-MCP 1 antisera obtained from two different rabbits. Antisera were heat inactivated (56°C, 30 min) before application.

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MCP1/JE ROLE IN IgA IMMUNE COMPLEX ALVEOLITIS IN RAT

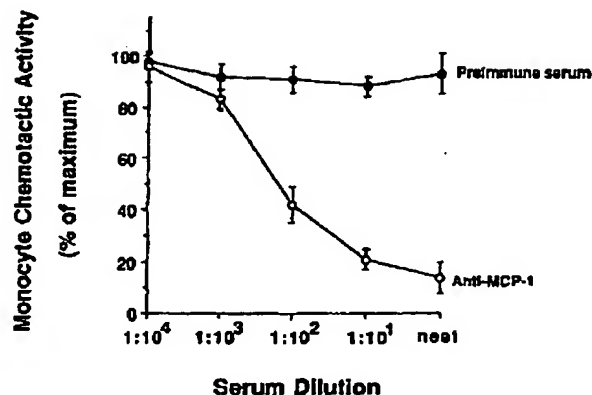


Figure 6. Neutralization of rMCP 1 monocyte chemotactic activity with anti-MCP 1 serum. Data (means \pm SEM; triplicate samples) are expressed as varying dilutions of antiserum vs monocyte chemotactic activity expressed as percentage of maximum response, where 100% is the response to 10^{-8} M MCP 1 (23 kDa). Sample chambers contained the indicated dilutions of heat-inactivated preimmune serum (\circ) or anti-MCP 1 serum (\bullet).

induced monocyte chemotactic activity secreted by rat endothelial cells. Rat pulmonary artery endothelial cells stimulated with human TNF- α expressed increased MCP 1 mRNA levels (data not shown) and secreted an 8- to 30-kDa monocyte-specific chemotactic activity (data not shown), which could be blocked in a dose-dependent manner with anti-rat MCP 1 (62% reduction in monocyte chemotaxis with a 1/100 dilution of anti-MCP 1 serum).

These data indicate that the anti-rat MCP 1 raised against 23-kDa rMCP 1 cross-reacts with the 18- and 21-kDa rMCP 1 species and that it specifically blocks rat rMCP 1 functional activity in vitro. Blockade of the 8- to 30-kDa monocyte-specific chemotactic activity secreted by TNF- α -stimulated rat pulmonary artery endothelial cells indicates that anti-MCP 1 neutralizes native rat MCP 1 activity. It should be noted that anti-MCP 1 had no blocking effect on FMLP (10^{-6} M)-mediated monocyte chemotaxis, zymosan-activated serum-mediated monocyte chemotaxis, or neutrophil chemotaxis (FMLP, 10^{-6} M; zymosan-activated serum) (data not shown).

Role of MCP 1 in pathogenesis of IgA immune complex-triggered alveolitis. Intravenous infusion of anti-MCP 1 antibody upon initiation of IgA immune complex-induced alveolitis resulted in a marked reduction in lung injury as quantified by pulmonary vascular permeability and pulmonary hemorrhage indices (Fig. 7; Table I). Analysis of BAL contents 4 h after anti-MCP 1 infusion revealed a nearly 80% reduction in retrievable mononuclear phagocytes compared with control animals (Table II). These data suggest that MCP 1 is required for the full development of IgA immune complex-induced alveolitis. The morphometric and cell retrieval data indicate that MCP 1 is required for maximum pulmonary monocyte/macrophage recruitment.

DISCUSSION

Based on in vitro studies that indicate that MCP 1/JE triggers monocyte chemotaxis and activation, and more recently, studies that have revealed either increased MCP 1/JE mRNA levels or immunoreactive MCP 1 monocyte chemotactic activity in atherosclerosis (11, 12), granulomas

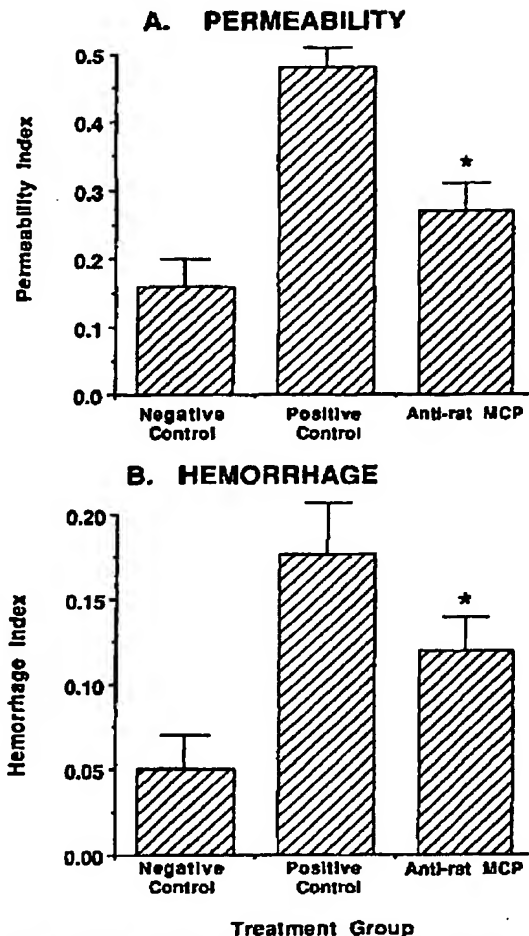


Figure 7. Blockade of IgA immune complex lung injury with anti-MCP 1 antibodies. Anti-MCP 1 (0.5 ml) was infused i.v. upon initiation of lung injury. Positive control rats were treated identically but received equivalent quantities of preimmune rabbit serum (0.5 ml) in place of anti-MCP 1. Negative control rats received intratracheal IgA anti-DNP/BSA but no i.v. Ag. Lung injury was quantitated by determining permeability indices (A) and hemorrhage indices (B) 4 h after instillation of anti-DNP/BSA as previously described (1-4). These data represent means \pm SEM of two experiments in which five rats per variable were employed. The data were analyzed by one-way analysis of variance with significance assigned for $p < 0.05$ (37). *Indicates a significant difference vs positive controls.

TABLE I
Morphometric analysis of IgA immune complex alveolitis: effect of anti-MCP 1 antibodies^a

Intervention	Mononuclear Phagocyte Influx		Alveolar Hemorrhage	
	M ϕ /40X HPF	p ^b	RBC/40X HPF	p ^b
A. Preimmune serum (0.5 ml) (positive control)	37 \pm 5		23 \pm 4	
B. Anti-MCP 1 serum (0.5 ml)	18 \pm 3	<0.05 vs A	11 \pm 3	<0.05 vs A
C. None (negative control)	13 \pm 3		4 \pm 1	

^a Immune complex alveolitis and anti-MCP 1 interventions were carried out as described in Figure 7.

^b One-way analysis of variance (37).

(13), and transplanted murine melanomas infiltrated with macrophages (30), it has been suggested that MCP 1/JE mediates monocyte/macrophage-rich pathologic processes. The present study suggests that MCP 1/JE

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MCP1/JE ROLE IN IgA IMMUNE COMPLEX ALVEOLITIS IN RAT

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TABLE II
Effect of anti-MCP 1 on alveolar macrophage retrieval in IgA
alveolitis^a

Treatment	Macrophages Retrieved	% of Total Cells	% Reduction ^b
A. None (negative control)	$4.2 \pm 0.62 \times 10^6$	93%	
B. Preimmune serum (0.5 ml; i.v.)	$9.8 \pm 0.83 \times 10^6$	89%	
C. Anti-MCP 1 serum (0.5 ml; i.v.)	$5.3 \pm 0.47 \times 10^6$	92%	79.7% vs B

^a Immune complex alveolitis and anti-MCP 1 interventions were carried out as described in Figure 7.

^b One-way analysis of variance (37).

may play an important role in the development of monocyte/macrophage-dependent IgA immune complex alveolitis in the rat. Infusion of antibodies directed against rat MCP 1 into rats with developing IgA immune complex-induced alveolitis resulted in a marked decrease in lung injury.

Critical to this study was to clone and express rat MCP 1. We chose the baculovirus/insect cell expression system because it has been successfully used to express other eukaryotic genes as nonfusion proteins (reviewed in Reference 29). Proteins produced in this expression system undergo post-translational processing and have been obtained in relatively large amounts (reviewed in Reference 29). We cloned rat MCP 1 cDNA from rat pulmonary artery endothelium stimulated with TNF- α because others have observed that TNF- α -stimulated human endothelial cells produce MCP 1 (26-28) and because we had previously detected markedly increased levels of rat MCP 1 mRNA in TNF- α -stimulated rat endothelium (M. L. Jones and J. S. Warren, unpublished data). Sf-21 cells infected with AcMCP 1 produced relatively large quantities of biologically inactive rat MCP 1. Solubilization and refolding of MCP 1 contained in Sf-21 cell lysate pellets yielded monocyte-specific chemotactic activity.

Yoshimura et al. recently cloned rat MCP 1 cDNA from Con A-stimulated rat spleen cells (18). The rat MCP 1 cDNA sequence published by Yoshimura et al. is in agreement with the sequence of rat JE genomic DNA reported by Timmers et al. (31). The deduced amino acid sequence of rat MCP 1 bears a strong degree of homology to both human and mouse MCP 1 (32-34). In the present study, SDS/PAGE analysis indicated that the rat MCP 1 species expressed in the baculovirus/insect cell expression system have m.w. of approximately 18 kDa, 21 kDa, and 23 kDa. A shift in the predominant protein species from 23 kDa (and to a lesser extent, 21 kDa), to 16- to 18-kDa species in tunicamycin-treated Sf-21 cell cultures suggests that the two larger species are glycosylated. A m.w. of 16 to 18 kDa agrees with the m.w. of unprocessed rat MCP 1 predicted by its open reading frame (18). Yoshimura et al. reported a 14-kDa monocyte-specific chemotactic activity in the supernatants of rat malignant fibrous histiocytoma cell lines (MFR 11) that express high levels of MCP 1 mRNA (18). A m.w. of 14 kDa approximates the predicted size of mature secreted (cleaved) rat MCP 1 in which the N terminus appears to be the glutamine located at position 24 (18). Amino acid sequence data from mature native human MCP 1 indicates that it starts with a glutamine at position 24. The predicted N-terminal amino acid sequence of unprocessed rat MCP 1 is hydrophobic, typical of a signal peptide, and consistent

with the observation that MCP 1 is a secreted protein.

As reported for various other proteins expressed in baculovirus insect cells (35), rat rMCP 1 was biologically inactive until it was denatured (in 8 M urea and 10 mM 2-ME) and then refolded by dialysis against cold HBSS containing 1 mM 2-ME. The refolded 21-kDa and 23-kDa rat MCP 1 species produced in this study were active in the monocyte chemotaxis assay at concentrations of approximately 35 ng/ml. Although the proportion of rat MCP 1 that is active could not be determined precisely, comparison with the reported potency of native human MCP 1 (optimal activity 10^{-9} M) suggests that only a small proportion of rat MCP 1 (<1%) is active (9, 10). It should be emphasized that this is only an approximation because native human MCP 1 and rat MCP 1 expressed using the baculovirus-insect cell system were not directly compared and because interspecies differences may influence activity quantitated by the monocyte chemotaxis assay. It is presently unknown exactly how polypeptides are produced or stored in a soluble form in vivo but several mechanisms may be operative. It is possible that insoluble aggregate formation is favored in insect cells that are overexpressing a foreign protein such as MCP 1. Aggregate formation may be caused by strong hydrophobic and/or ionic interactions among nascent peptides. Alternatively, normal protein folding may require so-called chaperones, proteins that regulate correct self-assembly of nascent peptides without themselves becoming incorporated (reviewed in Reference 36). Little is known about the secretion of native MCP 1 except that it appears to be processed via an N-terminal hydrophobic signal sequence (31-34). Finally, we cannot conclude from this study whether the monocyte chemotactic activity observed in the 21- and 23-kDa species (in contrast to the 18-kDa species) is a function of glycosylation or more efficient refolding.

Provision of MCP 1/JE-specific antibody was paramount to this study. As noted in Figure 5, rabbit polyclonal antibody raised against the 23-kDa rat rMCP 1 species reacted with the 18-kDa, 21-kDa, and 23-kDa baculovirus expression products as would be predicted if these represent variably glycosylated rat MCP 1 species. Anti-rat MCP 1 blocked MCP 1-triggered monocyte chemotaxis in a dose-dependent manner (Fig. 6). Finally, anti-MCP 1 selectively blocked monocyte-specific chemotactic activity secreted by TNF- α -stimulated rat endothelial cells. This observation, supported by the facts that TNF- α -stimulated rat endothelial cells, like human endothelial cells, express increased levels of MCP 1 mRNA (data not shown), that the monocyte-specific chemotactic activity is present in serum-free medium, and that the monocyte chemotactic activity has a m.w. between 8 and 30 kDa, provides compelling evidence that anti-MCP 1 recognizes and neutralizes native rat MCP 1.

The in vivo data indicate that MCP 1 is required for full development of IgA immune complex-induced pulmonary vascular leakage and hemorrhage. The lung lavage data (Table II) suggest that MCP 1 may play a role in recruitment of mononuclear phagocytes into the alveolar space. However, it is unclear whether this is a direct effect of local MCP 1 elaboration or a sequel to acute tissue injury per se. Attempts to measure MCP 1 activity in BAL fluid and serum were unsuccessful suggesting either that the amounts produced were below the level of detection by

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bioassay (10^{-10} M) or that MCP 1 is catabolized, complexed, or otherwise sequestered. It is also possible that MCP 1 is produced chiefly by the endothelium and thus cannot be measured by the assay methods in hand. The relative importance of MCP 1 as a chemotactic factor and as a monocyte/macrophage-activating factor remain to be determined in this model. Despite the issues to be addressed, these data indicate that MCP 1/JE plays an obligate role in the pathogenesis of IgA immune complex alveolitis in the rat.

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